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Post-exercise hypotension and skeletal muscle oxygenation is regulated by nitrate-reducing activity of oral bacteria

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Abstract

Post-exercise hypotension (PEH) is a common physiological phenomenon leading to lower blood pressure after acute exercise, but it is not fully understood how this intriguing response occurs. This study investigated whether the nitrate-reducing activity of oral bacteria is a key mechanism to trigger PEH. Following a randomized, double blind and crossover design, twenty-three healthy individuals (15 males/8 females) completed two treadmill trials at moderate intensity. After exercise, participants rinsed their mouth with antibacterial mouthwash to inhibit the activity of oral bacteria or a placebo mouthwash. Blood pressure was measured before, 1 and 2 hours after exercise. The microvascular response to a reactive hyperaemia test, as well as, blood and salivary samples were taken before and 2 hours after exercise to analyse nitrate and nitrite concentrations and the oral microbiome. As expected, systolic blood pressure (SBP) was lower (1 hour: -5.2 ± 1.0 mmHg; $P < 0.001$); 2 hours: -3.8 ± 1.1 mmHg, $P = 0.005$) after exercise compared to baseline in the placebo condition. This was accompanied by an increase of circulatory nitrite 2 hours after exercise (2h: 100 ± 13 nM) compared to baseline (59 ± 9 nM; $P = 0.013$). Additionally, an increase in the peak of the tissue oxygenation index (TOI) during the reactive hyperaemia response was observed after exercise (86.1 ± 0.6 %) compared to baseline levels (84.8 ± 0.5 %; $P = 0.010$) in the placebo condition. On the other hand, the SBP-lowering effect of exercise at was attenuated by 61% at 1 hour in the recovery period, and it was fully attenuated 2 hours after exercise with antibacterial mouthwash. This was associated with a lack of changes in circulatory nitrite ($P > 0.05$), and impaired microvascular response (peak TOI baseline: 85.1 ± 3.1 %; peak TOI post-exercise: 84.6 ± 3.2 %; $P > 0.05$). Diversity of oral bacteria did not change after exercise in any treatment. These findings show that nitrite synthesis by oral commensal bacteria is a key

- 24 mechanism to induce the vascular response to exercise over the first period of recovery
- 25 promoting lower blood pressure and greater muscle oxygenation.

26 **Introduction**

27 Post-exercise hypotension (PEH) is a common physiological response occurring in healthy and
28 hypertensive individuals which leads to a significant reduction of blood pressure over a few
29 hours after an acute bout of exercise (1, 2). However, how this intriguing physiological
30 response is elicited is not fully understood yet. Nitric oxide (NO) was originally suggested to
31 play a key role in PEH since it is well-established that exercise upregulates NO synthesis in
32 endothelial cells by stimulating endothelial NO synthase (eNOS) expression (3). However,
33 previous studies in humans concluded that PEH was not NO dependent. When endogenous
34 NO synthesis was blocked, by an intravenous infusion of the specific NOS inhibitor *N*^G-
35 monomethyl-L-arginine (L-NMMA), PEH was not affected (4, 5). Importantly, it was unknown
36 at that time that NO can be formed by another pathway which is independent of the L-
37 Arginine/NOS pathway (6). Thus, it is feasible that previous studies did not fully inhibit NO
38 synthesis as they intended to.

39 Currently, it is known that NO can also be formed by an oral nitrate/nitrite pathway. About
40 25% of the circulating nitrate in the human body is actively absorbed by the salivary glands
41 (7). Then, nitrate is secreted with saliva into the oral cavity where different species of oral
42 bacteria can reduce it to nitrite (8). Once swallowed, this nitrite is rapidly absorbed across the
43 upper gastrointestinal tract, increasing the bioavailability of nitrite in the circulation (6). In
44 the blood, nitrite can be reduced to NO by several enzymes and proteins leading to
45 vasodilation (9). Recent evidence in rats has shown that exercise can also enhance nitrite
46 reduction to NO by upregulating the enzyme xanthine oxidoreductase (XOR) (10). Thus, the
47 oral nitrate/nitrite pathway seems to complement the L-Argine/NOS pathway helping to
48 ensure that there is sufficient NO formation under different physiological situations (6). The

former mechanism relies on the status of the oral microbiome and previous studies have shown that the use of antibacterial mouthwash is an effective approach to inhibit the activity of oral bacteria that reduce nitrate to nitrite in the oral cavity (11-13). In addition, some of these studies found an increase in systolic blood pressure under resting conditions when antibacterial mouthwash was used for a few days (12, 13). This was associated with lower nitrite availability in saliva and plasma, and suggests that oral nitrite synthesis is a key mechanism in the regulation of blood pressure in humans. However, whether this is also important in PEH and exercise-induced muscle vasodilation is still unknown.

The main aim of this study was to investigate whether the oral nitrate/nitrite pathway is a key mechanism promoting PEH and skeletal muscle oxygenation after an acute bout of aerobic exercise at moderate intensity in healthy humans. We hypothesise that exercise will stimulate NO synthesis, which will be rapidly oxidized mainly to nitrate. During the recovery period, part of this nitrate, will be absorbed in the salivary glands, excreted into the mouth where anaerobic bacteria can reduce it to nitrite. This will lead to greater circulatory nitrite availability and higher PEH and skeletal muscle oxygenation levels concomitant with an improved PEH response. We also hypothesise that circulatory nitrite availability, PEH and skeletal muscle oxygenation will be significantly attenuated after exercise when inhibiting oral bacteria with antibacterial mouthwash.

Methods

Participants

The sample size of this study was estimated to detect differences of 3 mmHg in systolic blood pressure after using antibacterial mouthwash. Thus, twenty-two individuals in each group were required to have an 85% power at the 5% significance level. Participants were eligible to take part in the study if they did not: smoke; have a BMI > 30 kg·m⁻²; have hypertension,

dyslipidaemia or diabetes; suffer from an oral condition such as gingivitis or periodontitis or follow any treatment affecting the oral bacteria (mouthwash, tongue scrapes); take antibiotics within 3 months before the start of the study; and for females did not have irregular menstrual periods, less than or greater than 28 days over the last 3 months. All the participants provided written informed consent before starting the study. This study was approved by the Human Ethics Committee of Plymouth University (16/17-666) and registered on <http://www.clinicaltrials.gov> (NCT03904394).

Experimental protocol

In the first visit to the laboratory, participants performed an incremental treadmill test to assess their maximal aerobic capacity (VO_{2peak}). Respiratory gases (VO_2 , VCO_2 , RER) were measured breath-by-breath by a computerized gas analyzer (Jaeger Oxycon Pro, Germany). Heart rate was recorded using a heart rate monitor (Polar A300, Finland) during the test. Then, following a double-blind, randomized and cross-over design, participants visited the laboratory on two more occasions under fasting conditions (>3 hours). Upon their arrival at the laboratory, a cannula (Optiva IV catheter 20G) was inserted into an antecubital vein on the right arm for blood sampling. Then, a non-stimulated saliva sample was collected into a sterile falcon tube. Body mass, height and body fat (%) were also measured at the second and third visit. Following this, participants rested on a medical couch placed in a quiet room for 10 min, before the blood pressure was measured on the left arm using an electronic sphygmomanometer (ProBP 3400, Welch Allyn, US). A reactive hyperaemia test was also performed on the left arm. Levels of oxygenated haemoglobin (HbO_2) and deoxyhaemoglobin (HHb) on the left forearm (extensor digitorum) were continuously recorded using a near-infrared spectroscopy (NIRS) device (NIRO-200NX, Hamamatsu, Japan) at an output frequency

of 1 Hz. The NIRS probe was secured with an elastic tensor bandage wrapped around to minimize movement and light intrusion. After baseline measurements (2 min), an automatic pneumatic cuff (Hokanson E-20 AG101, USA) was inflated ~5 cm above the elbow for 5 min to an occlusion pressure of 200 mmHg. Then, inflation of the cuff was rapidly released and the NIRS measurements were continuously monitored for 5 more minutes. HbO₂ and HHb levels of the leg (rectus femoris) were also measured simultaneously during the reactive hyperaemia test using a second channel of the NIRS device and at the same frequency as the forearm. The NIRS probe was secured with an elastic tensor bandage wrapped around the leg to minimize light intrusion and movement. The average values during the 2 min baseline, 5 min occlusion, and 5 min recovery period were analysed.

Upon completion of all these measurements, participants performed a workout on a treadmill consisting of four sets of 7 min of running at 65% of VO_{2peak} interspersed with 3 min of passive recovery. After exercise, participants remained in the laboratory under resting conditions for 2 hours. Water was provided during this period, but food and other drinks were forbidden during the whole trial. Antibacterial mouthwash (Corsodyl, 0.2% chlorhexidine, GlaxoSmithKline, UK) or placebo (nitrite-free water with mint flavour) was provided to the participants at 1, 30, 60 and 90 min after exercise. They rinsed their mouth for 1 min on each occasion with the mouthwash. Blood pressure was measured following the same protocol as before exercise at 60 and 120 min of the recovery period. Blood and unstimulated saliva samples were taken before and at 120 min after exercise into a sterile tube. Saliva samples were rapidly centrifuged (14,000 rpm for 30 min at 4°C). The supernatant was collected into a sterile Eppendorf tube and stored at -80°C. The pellet in the bottom of the tube was also stored at -80°C to analyse the oral microbiome profile. Blood samples were also rapidly centrifuged (4,500 rpm for 10 min at 4°C). Plasma was collected into sterile Eppendorf tubes

and stored at -80°C. Participants came back one week later (washout period) to perform the same test, but with the other treatment (placebo or antibacterial mouthwash). Both tests were performed on the same day of the week and time (~2 hours) in order to reduce the effect of circadian variations.

Analyses

Plasma and salivary nitrate and nitrite

Plasma samples were mixed 1:1 with carrier solution and centrifuged (14,000 rpm for 20 min at 4°C) before injecting 50 µL into a dedicated High-Performance Liquid Chromatography (HPLC) system (ENO-30; EiCom, Kyoto, Japan). Standard curves were obtained for all measurements and used for quantitative measurements. Salivary nitrate and nitrite were measured as previously described (14).

Salivary and plasma pH, lactate, glucose

A single electrode digital pH meter (Lutron Electronic Enterprise Co Ltd., Model PH-208, Taiwan) was calibrated following the manufacturer's instructions and used to measure pH in saliva and plasma samples. Glucose and lactate were measured using a biochemistry analyser (YSI 2300 Stat Plus, YSI Life Sciences, USA).

Salivary buffering capacity

250 μ L of saliva was mixed with 750 μ L of HCl (0.0033 m/L) and shaken for 20 min. Then, salivary pH was measured using a single electrode digital pH meter (Lutron Electronic Enterprise Co Ltd., Model PH-208, Taiwan).

Oral microbiota

~35 mg of the saliva pellet was exposed to a 30 min lysozyme incubation step (37°C) prior to the DNA extraction. Salivary DNA was extracted using a DNeasy® Kit (Qiagen, Crawley, UK) following the manufacturer's instructions. PCR amplification of the 16S rRNA V1-2 region was carried out using universal 16S primers, 27F (5' AGA GTT TGA TCM TGG CTC AG 3') and 338R-I: (5'148 GCW GCC TCC CGT AGG AGT 3') and 338R-II (5' GCW GCC ACC CGT AGG TGT 3') (15). PCR products were purified using Agencourt® AMPure® XP (Beckman Coulter, High Wycombe, UK). High-throughput sequence analysis of the purified PCR products was done using a 318™ chip (LifeTechnologies™) on an Ion Torrent Personal Genome Machine (LifeTechnologies™) at the Systems Biology Centre in Plymouth University (UK). The 16S reads were processed by following the standard workflow of the DADA2 R package (version 1.10.1). In short, paired reads were filtered, trimmed and merged to produce a table of amplicon sequence variants (the counts of each unique sequence found in each sample). Taxonomy was then assigned to these sequences based on the Silva reference database (version 132) (16).

Statistical analysis

Results are presented as mean \pm standard error of the mean (SEM). Normal distribution of the sample was assessed using the Shapiro-Wilk test. Percentage change between pre and post-exercise oxygenation levels (rectus femoris) were compared using paired samples *t* test or Wilcoxon test when data was not normally distributed. A two-way repeated measures ANOVA was performed to assess the main effects and interactions between treatments

(placebo and antibacterial mouthwash) and time (pre and post-exercise). When the ANOVA revealed a significant interaction, specific differences were identified using individual comparisons according to Wei et al (17). Analysis was carried out using the SPSS software (SPSS Statistics, IBM® Version 24) and statistical significance was determined as $P < 0.05$.

For the analysis of the oral microbiome, the Kruskal-Wallis rank sum test was used to calculate differences in particular values (abundances of taxa or values of other continuous meta-data variables) between samples with different treatments. The Bonferroni correction was applied to adjust P values for multiple testing. Pearson's correlation was calculated between the abundances of taxa and measured continuous meta-data values, again applying the Bonferroni correction to P values. The ggplot2 package (version 3.1.0) (18) was used to produce boxplots and scatterplots.

Results

Twenty-three healthy and normotensive participants (table 1) successfully completed this study from May 2017 to April 2018. During the trial, one participant was excluded due to higher blood pressure readings. Biological samples (plasma and saliva) were taken from fifteen participants that gave signed informed consent.

Blood pressure

Systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean arterial blood pressure (MAP) results are shown in figure 1. SBP (placebo: 111.6 ± 2.0 mmHg; antibacterial mouthwash: 110.1 ± 1.6 mmHg), DBP (placebo: 65.7 ± 1.0 mmHg; antibacterial

185 mouthwash: 65.3 ± 0.8 mmHg) and MAP (placebo: 81.0 ± 1.1 mmHg; antibacterial
 186 mouthwash: 80.3 ± 0.9 mmHg) did not differ between treatments before exercise ($P > 0.05$).
 187 A time effect was found in DBP at 60 min post-exercise in the placebo condition (-2.4 ± 0.7
 188 mmHg, $P = 0.004$) that was attenuated by 29% with antibacterial mouthwash (-1.7 ± 1.0
 189 mmHg, $P = 0.07$) compared to pre-exercise values. However, no statistical differences ($P >$
 190 0.05) were found between treatments at 60 min post-exercise. Two hours after exercise, DBP
 191 was still lower in the placebo (-0.9 ± 0.7 mmHg), but not significantly compared to pre-
 192 exercise values ($P > 0.05$). There was a significant raise in DBP (1.8 ± 0.8 mmHg $P = 0.036$)
 193 between 60 and 120 min with antibacterial mouthwash reaching pre-exercise levels, however
 194 differences between both treatments were not evident at this point ($P > 0.05$).
 195 A significant reduction in SBP was observed at 60 min post-exercise with placebo (-5.2 ± 1.0
 196 mmHg; $P < 0.001$), which was attenuated by 62% (-2.0 ± 1.0 mmHg; $P = 0.004$) using
 197 antibacterial mouthwash. Differences between treatments were also statistically different (P
 198 $= 0.021$). SBP was still significantly lower (-3.8 ± 1.1 mmHg, $P = 0.005$) at 120 min post-exercise
 199 in the placebo compared to pre-exercise values. This response was fully inhibited with
 200 antibacterial mouthwash (0.3 ± 1.1 mmHg, $P > 0.05$) showing differences between treatments
 201 as well ($P = 0.026$).
 202 MAP decreased significantly at 60 min post-exercise with placebo (-3.3 ± 0.8 mmHg, $P <$
 203 0.001), and this was attenuated by 45% with antibacterial mouthwash, but there were no
 204 differences between treatments. MAP remained significantly lower (-1.9 ± 0.7 mmHg, $P =$
 205 0.014) at 120 min post-exercise in the placebo condition compared to pre-exercise values.
 206 This was also attenuated with antibacterial mouthwash (0.1 ± 0.8 mmHg, $P > 0.05$), although
 207 differences between treatments were not significant ($P > 0.05$).

208 ***Reactive hyperaemia test***

209 Figure 2 shows the tissue oxygenation index (TOI) (%) during the reactive hyperaemia test in
210 both treatments. No differences were found at baseline and during the occlusion of the
211 extensor digitorum between treatment and conditions (pre and post-exercise) (figures 2A and
212 2B). A higher peak TOI value (pre-exercise: 84.8 ± 0.5 %; post-exercise: 86.1 ± 0.6 %; $P = 0.010$)
213 after releasing the cuff pressure was found after exercise in the placebo condition only (figure
214 2B), but this was not evident with the antibacterial mouthwash treatment (pre-exercise: 85.1
215 ± 0.7 %; post-exercise: 84.6 ± 0.7 %; $P > 0.05$).

216 Exercise induced an increase of 3.0 ± 0.6 % of the TOI levels of the rectus femoris in the
217 placebo condition, but this response was significantly attenuated (0.8 ± 0.8 %; $P = 0.033$) with
218 antibacterial mouthwash (figure 2C).

219 ***Salivary and plasma nitrate and nitrite***

220 Figure 3 shows salivary and plasma nitrate and nitrite concentrations. Pre-exercise
221 concentrations did not differ between treatments. Although, no statistical differences were
222 found in plasma nitrite after exercise between conditions ($P = 0.071$), greater concentration
223 of plasma nitrite was found after exercise in the placebo condition compared to baseline
224 levels (pre-exercise: 59 ± 9 nM; post-exercise: 100 ± 13 nM; $P = 0.013$). Such elevation was
225 abolished with the antibacterial mouthwash treatment, and this was accompanied by a
226 significant reduction of salivary nitrite (pre-exercise: 129 ± 30 μ M; post-exercise: 9 ± 3 μ M; P
227 < 0.001) and an increase of salivary nitrate (pre-exercise: 252 ± 94 μ M; post-exercise: $649 \pm$
228 112 μ M; $P = 0.003$). After exercise, placebo salivary nitrite was significantly higher (65 ± 11
229 μ M; $P < 0.001$) while salivary nitrate lower (120 ± 26 μ M; $P < 0.001$) compared to antibacterial
230 mouthwash.

Salivary and plasma markers

Exercise did not induce significant changes in salivary or plasma markers in the placebo condition (figure 4), however antibacterial mouthwash significantly increased salivary lactate (placebo: 0.19 ± 0.03 mmol/L; antibacterial mouthwash: 0.48 ± 0.04 mmol/L; $P < 0.001$) and glucose (placebo: 0.04 ± 0.01 mmol/L; antibacterial mouthwash: 0.18 ± 0.02 mmol/L; $P < 0.001$) after exercise compared to placebo.

Oral microbiome

Figure 5 shows relative abundance of oral bacteria as represented by operational taxonomic units (OTU's) of the main salivary phylum (5A), genera (5B), and the 10 most abundant species (5C). No significant changes in any of these parameters occurred within the first 2 hours of the recovery period after exercise between either treatment. Alpha diversity did not differ after exercise between treatments either (figure 5D). A positive and significant association ($r = 0.435$; P value = 0.049) was found between the genus *Selenomonas* (figure 5E) and plasma nitrite after exercise in the placebo condition.

Discussion

The main finding of this study was that PEH and TOI levels as determined by NIRS were significantly attenuated when oral bacteria was inhibited with antibacterial mouthwash. This was associated with lower availability of salivary and plasma nitrite after exercise. This is the first evidence showing that the nitrate-reducing activity of oral bacteria is a key mechanism to induce the acute cardiovascular response to exercise during the recovery period in healthy individuals.

Our results challenge previous knowledge on nitrite metabolism suggesting that plasma nitrite concentration reflects the degree of eNOS activity following shear stress in healthy individuals (19, 20). While we found that plasma nitrite concentrations increased two hours after exercise in the placebo condition, this did not occur when antibacterial mouthwash was used. Importantly, mouthwash was only given after exercise so it did not limit the stimulatory effect of exercise on eNOS activity (21). However, the lack of increase in circulatory nitrite after exercise following the antibacterial mouthwash treatment suggests that the activity of oral commensal bacteria is essential for maintaining the circulatory levels of this anion during the recovery period after exercise. The vasodilatory effects of nitrite are well described by previous studies using intra-arterial infusions of this anion or dietary supplements (22, 23). However, this is the first evidence showing that the cardiovascular response to exercise during the recovery period is strongly influenced by the nitrate-reducing activity of oral bacteria.

Currently, it is still unclear how oral nitrite is absorbed into the circulation and how NO-like bioactivity occurs from circulatory nitrite. A number of distinct endogenous pathways are potentially involved in the reduction of nitrite to NO in the circulation. Particular interest has been focussed on red blood cells where deoxyhaemoglobin may facilitate the reduction of nitrite to NO (24). However, this hypothesis is controversial as the scavenging capacity of oxyhaemoglobin for NO makes it difficult to explain how nitrite-derived NO might escape red blood cells (25). Our results showing higher muscle oxygenation levels in the leg (rectus femoris) in the placebo condition after exercise in combination with greater nitrite concentration in plasma do not seem to support this hypothesis. Other mechanisms within the blood vessel such as XOR may contribute to the nitrite reductase capacity in the circulation (25). However, this hypothesis was based on experiments using blood vessels

representing a pathophysiological scenario, which may limit the application of such findings to other physiological conditions (25). S-nitrosylation of proteins is another potential mechanism linked to nitrite bioactivity. This is relevant to the current study given the well-known pro-oxidative stimulus induced by exercise (26-28). However, in this study we did not measure any NO species other than nitrite and nitrate. Other recent evidence conversely suggests that the blood pressure-lowering effect of circulatory nitrite is not mediated by NO (29). However, and in contrast to this, some of our findings are more likely to suggest that NO was involved in the vascular response after exercise. For instance, the higher peak value observed after the occlusion period during the reactive hyperaemia test has been associated with greater NO synthesis in previous studies (30, 31). This is also in contrast to the main findings of older studies that could not confirm the relationship between NO and PEH when the L-Arginine/NOS pathway was inhibited pharmacologically with NG-monomethyl-L-arginine (4, 5). However, these studies did not take into account that NO could still form through the oral nitrate/nitrite pathway (4, 5).

We expected to see higher concentrations of plasma nitrate after exercise due to enhanced NO synthesis in the endothelial cells, but this was not shown in this study. However, an interesting finding was the large accumulation of nitrate (158 %) observed after inhibiting oral bacteria with antibacterial mouthwash. In a previous study, using antibacterial mouthwash, we found that salivary nitrate increased by 31% under resting conditions in a group of vegetarians (32). This may suggest greater endogenous production of nitrate in the current study, which is probably due to NO formed during exercise. However, the lack of changes in salivary nitrate and nitrite in the placebo condition do not fully support this hypothesis, but we cannot discard that exercise may have upregulated other mechanisms to speed up the entero-salivary nitrate/nitrite pathway. For instance, it is unknown whether exercise may

301 increase the expression of sialin, the main nitrate transporter in the salivary glands (7), which
302 could potentially help to absorb circulatory nitrate more rapidly. Additionally, there is a lack
303 of studies looking at the effect of exercise on the nitrate-reducing activity of oral bacteria or
304 the saliva flow rate. We have recently found a positive and significant association between
305 the nitrate-reducing activity of oral bacteria and aerobic exercise performance in healthy
306 individuals (14). However, this finding was more associated with the chronic effect of exercise
307 training, and it remains to be elucidated whether acute exercise enhances the nitrate-
308 reducing activity of oral bacteria as well. On the other hand, unpublished data from our
309 laboratory show that exercise may be also effective in increasing saliva flow rate. This may
310 suggest a rapid turn-over of saliva that can help to increase circulatory nitrite more rapidly
311 after exercise. Overall, new studies are needed to elucidate all of these questions and to
312 enhance our knowledge on nitrate/nitrite metabolism and exercise.

313 Similar to previous studies, antibacterial mouthwash containing chlorhexidine potently
314 reduced (> 90%) salivary nitrite concentrations (11-13, 33). Some of these studies (12, 13),
315 but not all (33, 34), using the same approach, also reported a significant increase in SBP of
316 healthy and hypertensive individuals under resting conditions after using mouthwash for
317 three and seven days. However, the current study is the first evidence showing that acute
318 administration of mouthwash after exercise causes a significant attenuation of PEH, especially
319 SBP. This is an important finding for public health because mouthwash is commonly used by
320 the general population including patients with hypertension. Sales of antibacterial
321 mouthwash and dental rinse products in the US was estimated at \$1.4 billion in 2014 (35),
322 illustrating the wide spread use of such products.

323 We did not observe any statistically significant differences in the relative abundance of oral
324 bacteria in either treatment group after exercise, suggesting that neither the treatment nor
325 the acute exercise bout has influenced the oral microbiome. However, we cannot entirely
326 exclude the possibility of changes in the oral microbiome because 16S rRNA sequencing is not
327 able to differentiate between live and dead bacteria. Given the short time frame during which
328 the sampling occurred (2 hours post-exercise), it is possible that changes in the microbiome
329 composition were not yet evident. Recent data from our laboratory showed significant
330 alterations of the oral microbiome at all levels (phylum, genera and species) when
331 antibacterial mouthwash was taken twice daily for a week in healthy individuals (33).
332 Together, this points to differences in the acute and chronic effects of antibacterial
333 mouthwash on the oral microbiome. On the other hand, we found a significant increase in
334 salivary glucose and lactate after using antibacterial mouthwash. These changes did not cause
335 a significant reduction of salivary pH, although a trend in this way was evident. Importantly,
336 over the long term, these changes may increase the risk of periodontal disease (36), which
337 has been associated with cardiovascular disease (37).

338 This study has some limitations. First, 23 participants completed this study, but biological
339 samples (saliva and blood) were taken from 15 of them. While statistical differences were
340 found in some biological variables, such as plasma and salivary nitrate and nitrite, changes in
341 other variables, such as salivary pH and buffering capacity, were not statistically different
342 after the antibacterial mouthwash probably due to lower statistical power. Furthermore, this
343 study was performed in healthy and young individuals so further studies are needed before
344 translating our main findings to other populations, such as older individuals or hypertensive
345 patients.

In conclusion, this study shows the first evidence that PEH and skeletal muscle oxygenation after exercise are nitrite-dependent and this is regulated in part by the nitrate-reducing activity of oral bacteria. This was confirmed by inhibiting oral bacteria with antibacterial mouthwash which led to a significant reduction of salivary and plasma nitrite availability, and in turn, led to lower PEH and skeletal muscle oxygenation levels.

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Table 1: Physical and physiological characteristics of the participants (SD \pm SEM)

Gender (M/F)	15/8
Age (year/old)	27.4 \pm 1.2
Weight (kg)	72.2 \pm 2.9
Height (cm)	176 \pm 0.02
BMI	23.1 \pm 0.6
Body fat (%)	19.1 \pm 1.3
Heart rate (beats·min ⁻¹)	61 \pm 2
SBP (mmHg)	114.3 \pm 2.2
DBP (mmHg)	67.6 \pm 1.1
MAP (mmHg)	83.2 \pm 1.3
VO _{2peak} (mL·kg·min ⁻¹)	50.6 \pm 1.7

BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure;
MAP: mean arterial blood pressure; VO_{2max}: maximum oxygen uptake

Figure legends

Figure 1: Changes in diastolic (DBP) (A), systolic (SBP) (B) and mean arterial blood pressure (MAP) (C) at 60 and 120 min post-exercise compared to pre-exercise values (SD \pm SEM).

* Statistical differences $P < 0.05$ between pre and 60 min post-exercise values; ** Statistical differences $P < 0.05$ between placebo and antibacterial mouthwash; † Statistical differences $P < 0.05$ between 60 and 120 min post-exercise values.

Figure 2: Salivary (top) and plasma (bottom) concentration of nitrate and nitrite before and 2 hours post-exercise (SD \pm SEM).

* Statistical differences $P < 0.05$ between pre and post-exercise values; † Statistical differences $P < 0.05$ between antibacterial mouthwash and placebo.

Figure 3: Tissue oxygenation index (TOI) in the forearm (extensor digitorum) as measured by near-infrared spectroscopy during a hyperaemia reactive test before and 2 hours post-exercise after administering antibacterial mouthwash (2A) or placebo (2B) in the recovery period. Changes in tissue oxygenation index in the leg (rectus femoris) before and 2 hours post-exercise in both treatments (2C) (SD \pm SEM).

* Statistical differences $P < 0.05$ between pre and post-exercise values; † Statistical differences $P < 0.05$ between antibacterial mouthwash and placebo.

Figure 4: Salivary (top) and plasma (bottom) pH, lactate, buffering capacity (salivary only) and glucose before and 2 hours post-exercise (SD \pm SEM).

* Statistical differences $P < 0.05$ between pre and post-exercise values; † Statistical differences $P < 0.05$ between antibacterial mouthwash and placebo.

484 **Figure 5:** Relative abundance of the main salivary phyla (5A), genera (5B) and the 10 most
485 abundant species (5C) before (Pre) and after exercise (Post) following placebo (Plac) and
486 antibacterial mouthwash (AM) treatment. Alpha-diversity of salivary microbiota as
487 represented by Shannon-Index (5D) before and 2 hours post-exercise in both conditions
488 (placebo and antibacterial mouthwash). Pearson correlation ($r = 0.435$; $P = 0.049$) between
489 the relative abundance of *Selenomonas* and plasma nitrite 2 hours post-exercise in the
490 placebo condition (5E).